Cancer Gene Therapy (2002) 9, 725–736

www.nature.com/cgt

Evaluation of E1B gene-attenuated replicating adenoviruses for cancer gene therapy

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Gene-attenuated replication-competent adenoviruses are emerging as a promising new modality for the treatment of cancer. For the aim of improving adenoviral vectors for cancer gene therapy, we have constructed genetically attenuated adenoviral vectors with different combinations of *E1B* genes and investigated the possibility of enhanced oncolytic and replication effects of these engineered replication-competent adenoviruses. We show here that the cytolytic potency of each gene-attenuated replicating adenovirus differed significantly depending on the presence or deletion of *E1B 55* kDa and *E1B 19* kDa function. More specifically, among the constructed vectors (Ad-ΔE1B19, Ad-ΔE1B55, Ad-ΔE1B19/55, and Ad-wt), E1B 19 kDa–inactivated adenovirus (Ad-ΔE1B19) was the most potent against all tumor cells tested, inducing the largest-sized plaques and marked CPE. Further, cells infected with either Ad-ΔE1B19 or *E1B19/55* kDa–deleted adenovirus (Ad-ΔE1B19/55) showed complete cell lysis with disintegrated cellular structure, whereas cells infected with Ad-wt maintained intact cellular and nuclear membrane with properly structured organelles. TUNEL and DNA fragmentation assay also revealed that the Ad-ΔE1B19 or Ad-ΔE1B19/55 adenovirus-infected cells showed more profound induction of apoptosis in comparison to wild-type adenovirus-infected cells. The presence of *E1B 55* kDa gene was required for efficient viral replication and deletion of *E1B 19* kDa function in replicating adenovirus-induced apoptosis, leading to increased cytopathic effects. Moreover, Ad-ΔE1B19 adenovirus showed a better antitumor effect than other *E1B*-attenuated adenoviruses. Taken together, the replicating adenoviruses deleted in *E1B 19* kDa function may serve as an improved vector for anticancer gene therapy in combination with apoptosis-inducing modalities such as chemotherapeutic agents and radiation therapy. *Cancer Gene Therapy* (2002) **9,** 725–736 doi:10.1038/sj.cgt.7700494

Keywords: E1B mutant replicating adenovirus; E1B 19 kDa gene; E1B 55 kDa gene; cancer gene therapy

Selective replication of viral agents in tumors may lead to improved efficacy over nonreplicating agents due to the self-perpetuating nature of viral multiplication, lysis of the infected cancer cell, and secondary infections to adjacent cells. Genetically attenuated adenoviruses are being developed as selectively replicating antitumoral agents, and currently, a number of clinical trials with such viruses are ongoing to treat a variety of cancers.[1–3] Initial assessment of these trials demonstrates that gene-attenuated adenoviruses have some efficacy and exhibit safety and feasibility of treatment.[2,4] However, studies have also shown that adenovirus *E1B 55* kDa gene deletion conferring selectivity, i.e., deletion of *E1B 55* kDa could endow selective replication, frequently results in reduced potency of the virus in tumors.[5,6] To achieve sufficient antitumor efficacy, gene-attenuated replicating adenoviruses are currently being delivered by multiple injections of high-titer virus,[6] or in combination with chemotherapy.[7,8] Therefore, the development of a novel replication-selective agent with improved potency would be timely and could be applied more widely in anticancer gene therapy.

The *E1B* region encodes for two major proteins, *E1B 19* kDa and *E1B 55* kDa. *E1B 19* kDa protein is a potent apoptosis inhibitor and a homologue of *Bcl-2*–related apoptosis inhibitors.[9–12] The antiapoptotic effect of Ad *E1B 19* kDa was initially described by Rao et al,[13] who observed that *E1B 19* kDa inhibited adenovirus E1A protein-induced apoptosis. In several human tumor cell lines, Ad5 *E1B 19* kDa was shown to inhibit p53-induced apoptosis.[14,15] In addition, the antiapoptotic effect of *E1B 19* kDa has been demonstrated to be functionally equivalent to that of *Bcl-2* in terms of its ability to inhibit apoptosis induced by growth factor deprivation, treatment with gamma radiation, glucocorticoid, and various cytotoxic drugs.[16] Recently, combination studies with cisplatin or paclitaxel with adenovirus-mediated proapoptotic *Bax* gene induction have shown a synergistic increase of cytotoxicity against cancer cells.[17,18] However, adenoviral vectors used in these studies contained intact *E1B 19* kDa that can interfere with the antitumor effect by blocking the apoptotic pathway induced by a variety of chemotherapeutic agents. Therefore, the development of *E1B 19* kDa gene-attenuated adenovirus could greatly enhance cellular cytotoxicity caused by replicating adenovirus and subsequently could be used

Received May 27, 2002.

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alone, or in combination with cytotoxic agents inducing apoptosis-mediated cell death.

Besides interfering with the p53 function during viral infection, *E1B 55* kDa protein also performs other functions that are important in viral replication. During the early phase of infection, *E1B 55* kDa counteracts *E1A* functions that would otherwise lead to the stabilization of p53 and the induction of apoptosis.[14] In the late phase, *E1B 55* kDa, acting together with the *E4-orf6* gene product, is required for efficient cytoplasmic accumulation and translation of adenoviral mRNA.[19–21] This is accompanied by shutting off of host mRNA nuclear export and host protein synthesis. Gene-attenuated replicating adenovirus, ONYX-015 (originally named dl1520), has a deletion in the *E1B* transcription unit that removes the gene for *E1B 55* kDa. This agent has been tested in phases I and II clinical trials for head and neck cancer, pancreatic carcinoma, ovarian cancer, colorectal carcinoma, non small cell lung carcinoma, and oral dysplasias.[2,3] Despite clear evidence of antitumoral activity in some patients, treatment with ONYX-015 as a single agent has not produced classically defined tumor responses. Thus, although targeting the p53 pathway produces specific cytotoxicity against the *p53* mutated cancer cells, the imperfection of the vector system may limit the therapeutic value in *in vitro* and in *in vivo*. Previously, we have shown that the *E1B 55* kDa–deleted recombinant adenovirus vector (YKL-1) exhibited the tumor-selective replication, but showed reduced cytopathic effect (CPE) in several tumor cell lines.[22] The reduced potency of dl1520 or YKL-1 may be due to a loss of an important function of *E1B 55* kDa that is involved in efficient viral replication such as viral mRNA nuclear transport and accumulation in the cytosol.

Recently, the *E1B 19* kDa–deleted adenovirus has been developed, showing increased induction of apoptosis and opening the possibility of enhancing potency of genetically attenuated replicating adenoviruses that can be ultimately used in a clinical setting.[23] In that context, it would be highly desirable to potentiate the cell killing of replicating adenoviruses by enhancing the viral-mediated cell lysis as well as the oncolytic effects of efficient viral replication. Based on these considerations, attempts to engineer *E1B 19* kDa and *E1B 55* kDa genes have been sought to advance the therapeutic value of replicating adenovirus for cancer gene therapy. However, to date, a systematic comparison and assessment of the therapeutic values of adenoviral *E1B 19* kDa and *55* kDa gene-modified adenoviral vectors have not been accomplished. In this study, we have constructed genetically attenuated adenoviral vectors with different combinations of *E1B* genes and have investigated the possibility of enhanced oncolytic and replication effects of these genetically engineered replicating adenoviruses.

Materials and methods

Cell lines and cell culture

All cell lines, with the exception of Hep3B, were cultured in DMEM supplemented with 10% fetal bovine serum and penicillin–streptomycin (100 IU/mL). The Hep3B cell line was maintained in modified Eagle's medium supplemented with 10% fetal bovine serum and penicillin–streptomycin (100 IU/mL). HEK293 (human embryonic kidney cell line expressing the adenoviral E1 region), liver cancer cell line (Hep3B), lung cancer cell lines (A549 and H460), cervical cancer cell line (C33A), and brain cancer cell line (U343) were purchased from the American Type Culture Collection (ATCC, Manassas, VA).

Construction of gene-attenuated adenoviruses

To generate the *E1B19/55* kDa–deleted recombinant adenovirus (Ad-ΔE1B19/55), the *E1A* region from nucleotide 343–1657 bp was amplified by polymerase chain reaction (PCR) with the following primer set: 5′-TTATTGGATCCTTTGTCTAGGGCCGCGGG-3′ as the sense primer and 5′-CCAGGATCCAGATCTCCCCATTTAACACGCCATGC-3′ as the antisense primer. The adenoviral shuttle vector, pXC1 (Microbix, Ontario, Canada), containing the *E1* region of adenovirus type 5, was used as a template. The PCR product containing *E1A* with *Bam*HI site (underlined) at both 5′ and 3′ ends and *Bgl*II (underlined) at the 3′ end was digested with *Bam*HI and then subcloned into the *Bgl*II site of pCA14 (Microbix), generating a pCA14-E1A shuttle vector. The nucleotide sequence of the amplified PCR product was verified using an ABI PRISM377 automatic DNA sequencer. The pCA14-E1A shuttle vector was linearized by *Xmn*I digestion. The adenovirus vector vmdl324Bst (obtained from Dr Verca at the University of Fribourgh, Switzerland) containing the Ad5 genome deleted in the *E1* region (281–4640 in nucleotide of Ad5) and the *E3* region (28596–30470 in nucleotide of Ad5) was linearized with *Bst*BI digestion. The linearized pCA14-E1A was cotransformed into *Escherichia coli* BJ5183 together with the *Bst*BI-digested vmdl324Bst for homologous recombination.[47] To verify the respective homologous recombinants, the plasmid DNA purified from overnight *E. coli* culture was digested with *Hin*dIII, and the digestion pattern was analyzed. The proper homologous recombinant adenoviral plasmid DNA was digested with *Pac*I and transfected into 293 cells to generate the *E1B*-deleted adenovirus, Ad-ΔE1B19/55.

To construct the *E1B19* kDa–inactivated adenovirus (Ad-ΔE1B19), pXC1 was digested with *Xba*I and *Kpn*I, and subsequently subcloned into the *Xba*I and *Kpn*I sites of pSP72 cloning vector, generating pSP72/E1B19. Then, the site-directed mutagenesis (Stratagene, La Jolla, CA) in pSP72/E1B19 was accomplished by PCR amplification using 5′-GTTACATCTGACCTCCTG***TAG***GCTAGCGAGTGTTTGGAAG-3′ as the sense primer and 5′-CTTCCAAACACTCGCTAGCCTACAGGAGGTCAGATGTAAC-3′ as the antisense primer. The primers were designed to create a stop codon (bold, italicized) and a new *Nhe*I site (underlined) in the region of start codon of *E1B 19* kDa. The new *Nhe*I site was introduced to confirm the mutation of PCR product. The mutated region was verified using an automatic sequencer. The resulting mutated plasmid pSP72/ΔE1B19 was digested with *Xba*I and *Kpn*I, and subcloned into the *Xba*I and *Kpn*I sites of pXC1. Then, homologous recombination was induced in *E. coli* BJ5183 with vmdl324Bst as described above. Replication-incompetent adenovirus (Ad-ΔE1), *E1B 55* kDa–deleted adenovirus (Ad-ΔE1B55; YKL-1), and wild-type recombinant adenovirus (Ad-wt)

were also prepared.[22] All viruses were propagated in HEK293 cells and purified according to standard methods.[22] Viruses used in this study are shown in Figure 1, and Ad-ΔE1 and Ad-wt were used as controls for E1B gene-attenuated adenoviruses.

PCR analysis of E1B mutant adenoviruses

U343 cells were infected with Ad-ΔE1, Ad-ΔE1B19/55, Ad-ΔE1B19, Ad-ΔE1B55, or Ad-wt at a multiplicity of infection (MOI) of 10. After 2 days postinfection, viral DNA was isolated with a genomic isolation kit (Qiagen, Santa Clarita, CA). PCR was accomplished using three kinds of E1 region–specific primer sets corresponding to *E1A*, *E1B 19*, and *E1B 55* kDa, and the expected sizes of PCR products were 479, 429, and 338 bp, respectively. PCR product fragments were then resolved on a 1.8% agarose gel and visualized by UV fluorescence.

Western blot analysis of Ad E1A and E1B 19 kDa protein expression

Cells were lysed in 50 mM Tris–HCl (pH 7.6), 1% Nonidet P-40 (NP-40), 150 mM NaCl, and 0.1 mM zinc acetate in the presence of a protease inhibitors. Protein concentration was determined using the Lowry method (Bio-Rad, Hercules, CA). Ten micrograms of each sample was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The gels were then electroblotted onto a nitrocellulose membrane. Membranes were blocked in TBST (50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.2% Tween 20) containing 5% dry milk overnight at 4°C. After blocking, membranes were incubated with a rabbit polyclonal IgG against *E1A*, a mouse monoclonal IgG against *E1B 19* kDa, or a mouse monoclonal IgG against *E1B 55* kDa (a generous gift from Dr Thomas Dobner in University of Regensburg, Germany). The secondary antibodies were goat anti–rabbit IgG horseradish peroxidase (HRP) or goat anti–mouse IgG HRP. Finally, the blots were developed using enhanced chemiluminescence (ECL) (Amersham Pharmacia, Arlington Heights, IL).

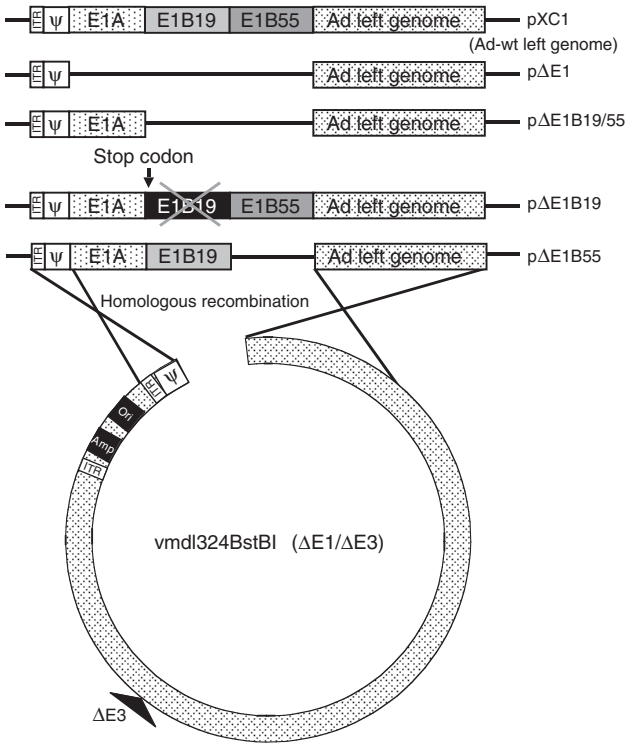

Figure 1 Schematic representation of three *E1B* mutant adenoviruses, Ad-ΔE1B19, Ad-ΔE1B55, and Ad-ΔE1B19/55, along with Ad-ΔE1 and Ad-wt. Ad-ΔE1 has the whole E1 region deleted; Ad-ΔE1B19 contains the normal E1A and E1B55 kDa, but is E1B19 kDa translation initiation codon–mutated; Ad-ΔE1B55 contains the normal E1A and E1B19 kDa, but is E1B55 kDa-deleted; Ad-ΔE1B19/55 contains E1A, but is E1B19 kDa- and E1B55 kDa-deleted.

CPE assays

To evaluate the CPE, $2-8\times10^4$ cells were plated onto a 24-well plate at about 30–80% confluence, and then infected with Ad-ΔE1, Ad-ΔE1B19/55, Ad-ΔE1B19, Ad-ΔE1B55, or Ad-wt at an MOI of 0.1–10. Their killing effect was monitored daily under a microscope. At the moment that cells infected with any one of the virus at an MOI of 0.1 exhibited complete cell lysis, cells on the plate were then stained with 0.5% crystal violet in 50% methanol.

For viral replication assays, cells were grown as described for the CPE assays but were subsequently infected at an MOI of 1 with either *E1B* mutant or control adenovirus (Ad-ΔE1 or Ad-wt). Forty-eight hours postinfection, both cells and supernatant were collected for virus titration analysis. Cell lysates underwent three cycles of freezing and thawing, and serial dilutions of supernatants and lysates were tittered on 293 cells.

Electron microscope (EM) cytology

A549 cells were infected with Ad-ΔE1B19 or Ad-wt at an MOI of 10. Four days postinfection, cells were gently trypsinized and pelleted for 5 minutes at 3000 rpm in a microcentrifuge tube. After washing with phosphate-buffered saline (PBS), pellets were fixed for 4 hours at 4°C in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) containing 2% sucrose and 1 mM calcium chloride. They were then postfixed with 1% OsO_4 in 0.1 M cacodylate–HCl, pH 7.4, for 1 hour. The samples were dehydrated in a gradient series of ethanol, embedded in Epon812, and examined using an EM.

Virus production assay

To assay for viral growth, cells were plated onto a six-well plate at about 70% confluence, and then infected with Ad-ΔE1, Ad-ΔE1B19/55, Ad-ΔE1B19, Ad-ΔE1B55, or Ad-wt at an MOI of 1. At various times of incubation at 37°C, supernatant and cells were collected and freeze-thawed three times to release the viruses. Supernatant and monolayer samples were assayed by plaque assay to determine the virus titer.

Plaque development assay

Plaque development assay was carried out on 293, Hep3B, and U343 cell lines as described previously.[48] Briefly, monolayers of each cell line were infected with serial diluted adenoviruses in six-well plates. After 2 hours of incubation

at 37°C, the infected cells were overlaid with 4 mL of 0.7% SeaPlaque agarose in DMEM with 5% fetal bovine serum. Following 7–9 days of incubation, agarose overlay was removed after soaking with 10% trichloroacetic acid for 30 minutes, and the remaining cells were stained with 0.5% crystal violet in 50% methanol.

TUNEL assay

Apoptosis was also analyzed by terminal deoxynucleotidyl transferase (TdT)–mediated dUTP nick end labeling (TUNEL) assay as described previously.[22] Briefly, A549 cells were plated on a chamber slide 1 day prior to infection, and treated with Ad-ΔE1B19/55, Ad-ΔE1B19, Ad-ΔE1B55, or Ad-wt at an MOI of 2. After 4 days of infection or treatment with 1 μM camptothecin (CPT), infected cells were rinsed with PBS. Samples were then processed according to the instructions in the ApopTag kit (Oncor, Gaithersburg, MD) for the detection of cleaved deoxyribonucleic acid *in situ* using the TdT-mediated deoxyuridine 5′-triphosphate-biotin nick end labeling (TUNEL) method. The apoptotic cells were counted under magnification ($\times$400) in 10 selected fields. More than 2000 cells were counted to calculate the percentages of TUNEL-positive cells (apoptotic cell ratio).

DNA fragmentation assay

Confluent cultures were treated with CPT at 1 μM or infected with Ad-ΔE1, Ad-ΔE1B19, Ad-ΔE1B55, Ad-ΔE1B19/55, or Ad-wt at an MOI of 10. Cells were harvested and suspended in a lysis buffer (10 mM Tris–HCl, pH 8.0, 100 mM NaCl, 25 mM EDTA, 0.5% SDS, and 100 μg/mL Proteinase K) for 20 hours at 37°C. DNA was extracted with a mixture of phenol and chloroform, precipitated by ethanol, dried, and dissolved in a TE buffer. DNA samples were analyzed by electrophoresis on a 2% agarose gel containing ethidium bromide (0.5 μg/mL) and then visualized under a UV illuminator.

Antitumor effect of E1B mutant adenoviruses in vivo

The antitumor effect of the following viruses was tested *in vivo*: Ad-ΔE1, Ad-ΔE1B19, Ad-ΔE1B55, Ad-ΔE1B19/55, and Ad-wt. Five- to 6-week-old female nude mice were purchased from Charles River (Yokohama, Japan). Tumors were implanted on the abdomen of each animal by subcutaneous injection of 10^7 tumor cells in 100 μL of PBS. Tumor growth was monitored on a 2- to 3-day interval by measuring the length and width of the tumor with a caliper. Tumor volume was calculated using the following formula: volume$=0.523Lw^2$. When tumors reached a range of 60–70 mm^3, animals were randomized into five groups of six animals each. First day of treatment was designated as day 1. Adenoviruses were administered intratumorally (5×10^8 PFU per tumor in 50 μL of PBS) on days 1, 3, and 5. Tumor growth delay was assessed by taking measurements on a 2- to 3-day interval. When the tumors reached the maximum acceptable size, mice were sacrificed and their tumors were excised and analyzed histologically (hematoxylin and eosin). Tumor responses to each treatment were compared by Mann-Whitney test.

Results

Generation and characterization of E1B mutant adenoviruses

To characterize and compare the *E1B* mutant adenoviruses' potential for cell killing effect, three *E1B*-attenuated adenoviruses (Ad-ΔE1B19, Ad-ΔE1B55, and Ad-ΔE1B19/55) were developed as described in Materials and Methods. First, Ad-ΔE1B19 was designed to be deficient of the *E1B 19* kDa protein by inserting a stop codon in the region of *E1B 19* kDa translation initiation codon. Second, Ad-ΔE1B55, as described previously, lacked the *E1B 55* kDa gene.[22] Finally, Ad-ΔE1B19/55 was designed to express neither the *E1B* 19 kDa nor the *55* kDa proteins due to the deletion of both genes. All three constructs were created in adenovirus subtype 5 dl324 background (Fig 1).

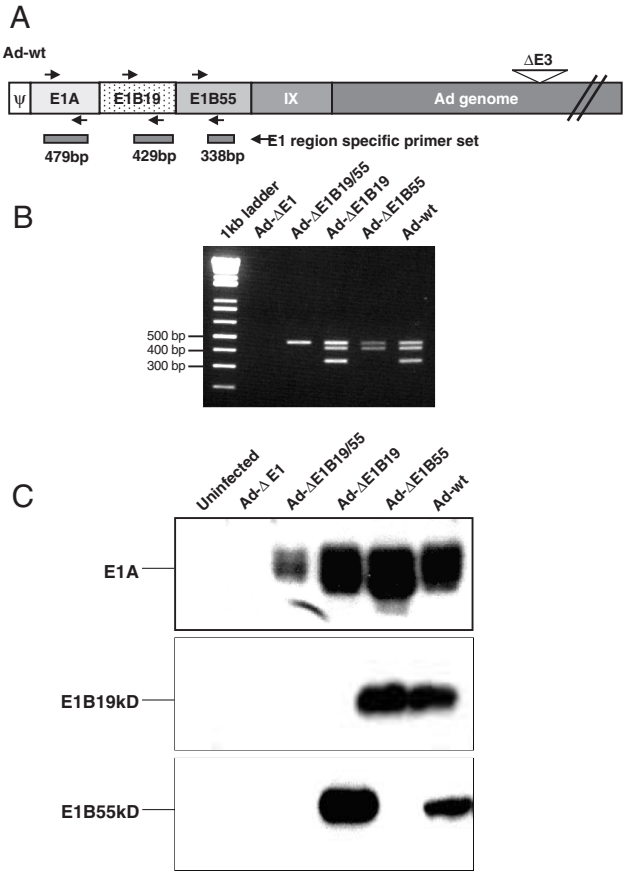

Figure 2 A: The *E1* region–specific primer sets corresponding to *E1A*, *E1B19* kDa, and *E1B55* kDa are shown below the diagram of *E1* region of adenovirus genome, and each expected size of PCR products is 479, 429, and 338 bp, respectively. **B:** PCR product analysis of *E1B* mutant adenoviruses. Left marker (GIBCO, Gaithersburg, MD), 1-kb DNA ladder. The presence of each PCR product verified the presence of the gene deletion. **C:** Detection of the *E1A*, *E1B 19* kDa, and *E1B* 55 kDa protein by Western blot analysis. Twenty-four hours after infection, total protein from A549 cells infected with Ad-ΔE1, Ad-ΔE1B19, Ad-ΔE1B55, Ad-ΔE1B19/55, or Ad-wt at a dose of 10 MOI was analyzed with anti-E1A, anti-E1B19, or anti-E1B55 antibody as described in Materials and methods.

Gene constructs of each generated adenoviruses were verified by PCR using appropriate primer sets amplifying each *E1* region corresponding to *E1A*, *E1B 19* kDa, and *E1B 55* kDa genes (Fig 2A). The U343 brain tumor cell line was infected with each *E1B* mutant adenovirus at an MOI of 10 along with Ad-ΔE1 and Ad-wt as a negative and a positive control, respectively. After 2 days postinfection, viral DNA was isolated and subsequently used for PCR amplification using three *E1*-specific primer sets. As expected with the *E1A*-specific primer set, PCR generated the DNA fragment of 489 bp from all samples except Ad-ΔE1–infected U343 cells (Fig 2B). Deletion of *E1B 55* kDa gene in Ad-ΔE1B55 genome was confirmed by PCR amplification using *E1B 55* kDa–specific primer set, resulting in no PCR product of 338 bp. However, *E1A* (479 bp) and *E1B 19* kDa (429 bp) DNA fragments were amplified with *E1A*- and *E1B 19* kDa–specific primers, respectively, indicating that Ad-ΔE1B55 had a deletion of *E1B 55* kDa gene but that the E1A and *E1B 19* kDa regions were still intact in the genome. The Ad-ΔE1B19/55 genome structure was verified by PCR amplification, generating only a *E1A*-specific PCR product (479 bp) but not *E1B 19* kDa and *E1B 55* kDa products. This result indicated that *E1B 19* kDa and *E1B 55* kDa genes were properly deleted in Ad-ΔE1B19/55 genome. When Ad-ΔE1B19–infected U343 cell–derived genomic DNA was amplified by three *E1* region-specific primer sets, three products corresponding to *E1A*, *E1B 19* kDa, and *E1B 55* kDa were obtained. Ad-ΔE1B19 genome, which only has a change of an initiation codon at the stop codon but retained all coding sequences of *E1B 19* kDa region, resulted in amplification of all three PCR-amplified DNA fragments.

To verify the gene composition of the three *E1B* mutant adenoviruses, *E1A*, *E1B 19* kDa, and *E1B 55* kDa protein

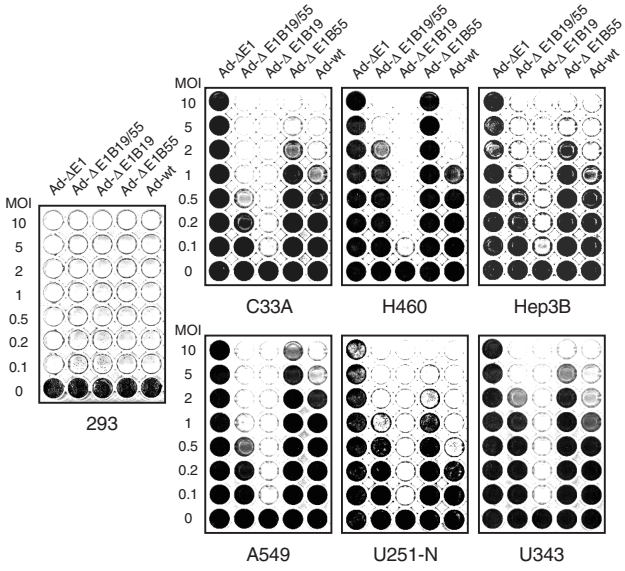

Figure 3 CPE effects of replicating adenoviruses *in vitro*. Monolayer of cells was infected with different viruses, as indicated. Replication-incompetent adenovirus Ad-ΔE1 and wild-type adenovirus Ad-wt served as controls. When cells infected with any one of adenoviruses at an MOI of 0.1 were completely lysed, cells remaining on the plates were fixed and stained with crystal violet.

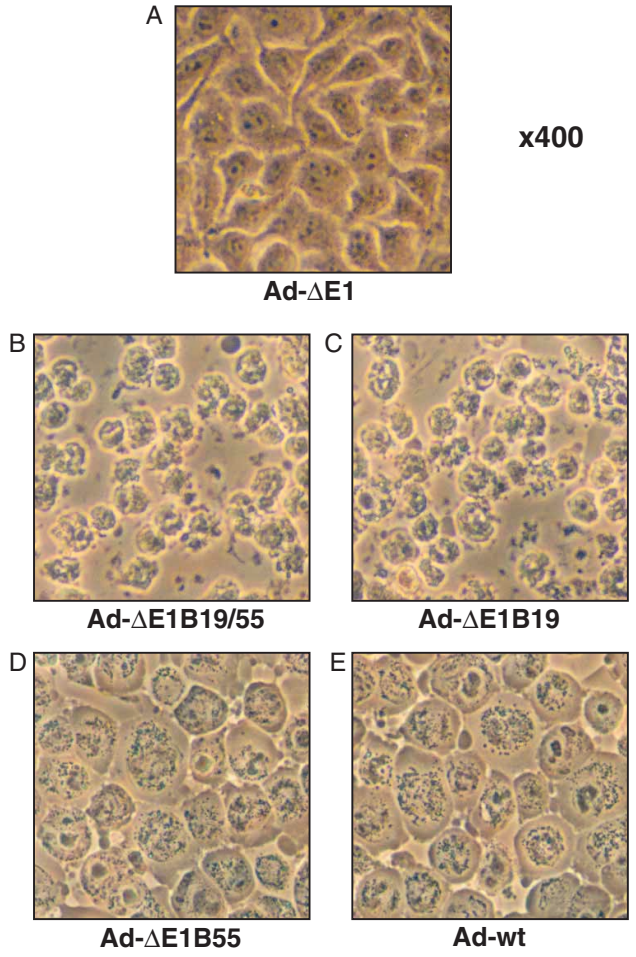

Figure 4 Morphology of U343 cells infected with Ad-ΔE1 **(A)**, Ad-ΔE1B19/55 **(B)**, Ad-ΔE1B19 **(C)**, Ad-ΔE1B55 **(D)** or Ad-wt **(E)**. Infections were performed at an MOI of 10 as described in Materials and methods. Micrographs of infected cells were taken at 4 days postinfection.

expressions were evaluated by immunoblotting with anti-E1A, anti–E1B 19 kDa, anti–E1B 55 kDa antibodies. As shown in Figure 2C, no expression of *E1B 19* kDa was found in cell lysates derived from cells infected with Ad-ΔE1B19 or Ad-ΔE1B19/55 adenoviruses in contrast to those infected with Ad-ΔE1B55 or Ad-wt adenoviruses. The expression of *E1B 55* kDa was detected in cells infected with Ad-ΔE1B19 or Ad-wt in contrast to those infected with Ad-ΔE1B55 or Ad-ΔE1B19/55. Next, as expected, *E1A* protein was well expressed in cells infected with each *E1B*-attenuated adenovirus or wild-type adenovirus but not with Ad-ΔE1 adenovirus. These results confirmed that, as intended, all three *E1B* mutant adenoviruses possessed proper *E1B* gene organization and expression pattern.

Evaluation of cell killing effect of E1B mutant adenoviruses

To quantitatively assess adenovirus replication–dependent cell killing effects, cell cytotoxicity of each mutant adenovirus was analyzed by an *in vitro* CPE assay. Cells were grown to 50–80% confluence and then infected at an MOI of 0.1–10. For control, cells were infected in parallel

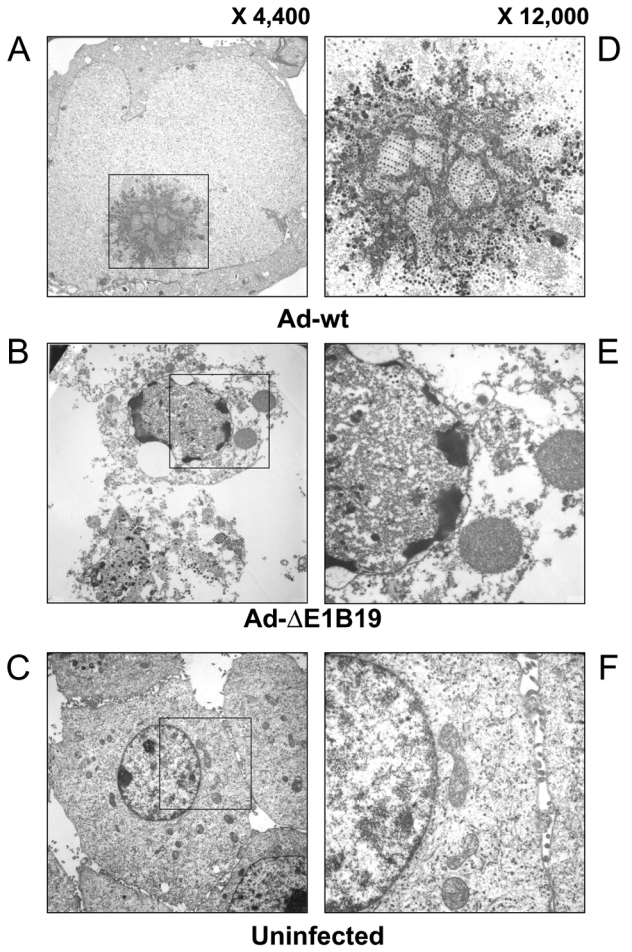

Figure 5 Electron micrographs of uninfected Ad-wt **(A,D)** or Ad-ΔE1B19 **(B,E)** and uninfected **(C,F)** A549 cells at 4 days post-infection. Cells were infected at an MOI of 10 as described in Materials and methods. Typical morphologies are shown. Representative low power (×4,400) **(A–C)** and high power (× 12,000) **(D–F)** images are shown.

with nonreplicating adenovirus Ad-ΔE1 and wild-type adenovirus. As shown in Figure 3, Ad-ΔE1B19 exerted marked CPE against all tumor cell lines tested. The cell lysis capacity of Ad-ΔE1B19 was about 10–100 times greater than either Ad-ΔE1B55 or Ad-ΔE1B19/55, and about 5–20 times greater than wild adenovirus Ad-wt. At similar MOIs, the decrease in viable cells was significantly more evident in cells infected with Ad-ΔE1B19 than in those with any other *E1B* mutant adenoviruses. These results demonstrated that the loss of *E1B 19* kDa enhanced adenoviral lytic potency over that of other *E1B* mutant adenoviruses as well as that of wild type. In regards to comparison of Ad-ΔE1B19/55 to Ad-ΔE1B55 adenovirus, Ad-ΔE1B19/55 adenovirus exerted higher potency of cell killing over Ad-ΔE1B55 adenovirus. More specifically, Ad-ΔE1B19/55 showed about 20 times greater cytotoxicity than Ad-ΔE1B55 adenovirus in A549 cells, and about 2–5 times greater potency in C33A, H460, Hep3B, U251N, and U343 cells. Overall, these four replication-competent adenoviruses exerted varied CPE in the order of Ad-ΔE1B19>Ad-wt>Ad-ΔE1B19/55>Ad-ΔE1B55.

Different rates of cell death were also observed morphologically. U343 cells infected with Ad-ΔE1, Ad-ΔE1B19, Ad-ΔE1B55, Ad-ΔE1B19/55, or Ad-wt adenovirus at an MOI of 10 were observed by light microscope up to 7 days. Ad-ΔE1 adenovirus–infected cells showed no CPE during the observed time course. Cells infected with Ad-ΔE1B19 exhibited obvious CPE and cell lysis by 2 days postinfection, whereas cells infected with other Ad *E1B* mutants or with Ad-wt were nearly all intact until 3 days postinfection, and cell death progressed from days 4 to 7. A representative example of these cells at 4 days postinfection is shown in Figure 4. Cell death process by infection with Ad-ΔE1B19 was faster than that of any other adenoviruses.

It has been noted that cells infected with *E1B 19* kDa–attenuated adenoviruses, Ad-ΔE1B19 and Ad-ΔE1B19/55, showed distinct morphology in the CPE in that the cell membrane was almost completely lysed and cellular structure was severely disintegrated (Fig 4). In comparison, cells infected with Ad-ΔE1B55 or Ad-wt showed typical adenovirus-mediated cell lysis, having cells rounded up and detached from the plastic dishes into floating cells and forming grape-like clusters. The floating cells were highly light-refractive and majority of these cells were viable as judged by viability studies using trypan blue exclusion assay.

The morphological difference observed was further examined by EM. At 4 days postinfection at an MOI of 10, majority of A549 cells infected with Ad-ΔE1B19 adenovirus were lysed, whereas nearly all Ad-wt–infected cells were intact. The nuclei in the Ad-wt–infected cells were extremely swollen, occupying nearly the entire cell (Fig 5A). Nuclei were packed with viruses but the nuclear membrane still appeared to be intact. In some cells, crystals of viruses were observed (Fig 5D). All intracellular organelles in Ad-wt–infected cells were intact and retained proper structure. However, cells infected with Ad-ΔE1B19 were completely lysed. The cellular membrane and nuclear membranes were severely disintegrated and not distinct (Fig 5B). The intracellular organelles were also hardly distinguished, and some showed ballooning degeneration. Viral particles were observed to spread out in the cytoplasm.

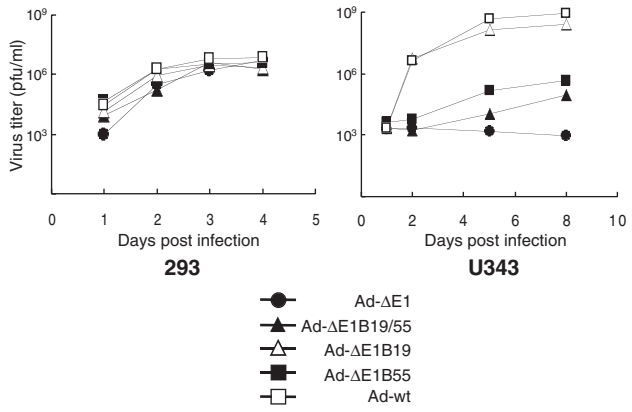

Figure 6 Virus production assay. Monolayers of 293 and U343 cells were infected with Ad-ΔE1, Ad-ΔE1B19/55, Ad-ΔE1B19, Ad-ΔE155, or Ad-wt at an MOI of 1. The virus present in cell and supernatant was extracted at the indicated days postinfection, and titers were determined by plaque assay.

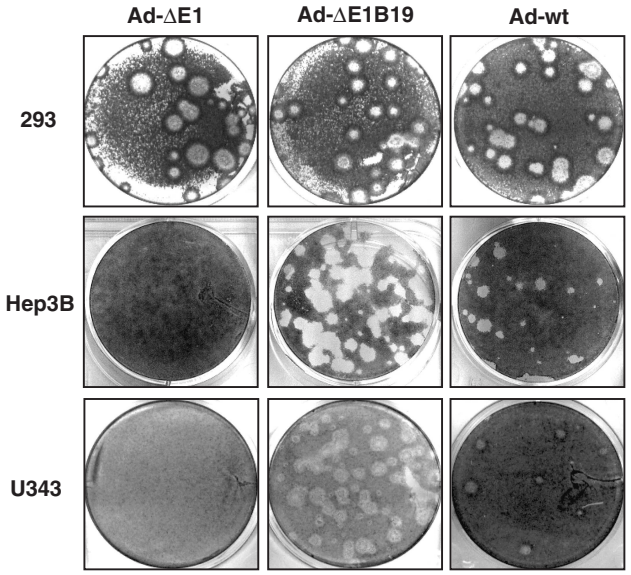

Figure 7 Plaque morphology of Ad-ΔE1, Ad-ΔE1B19, and Ad-wt on 293, Hep3B, and U343 cells. After a 2-hour adsorption period, plates were overlaid with agarose and incubated. At 7–9 days postinfection, the agarose overlay was removed, stained with crystal violet, then photographed. Ad-ΔE1B19 lacking E1B 19 kDa mutant adenovirus developed larger and more number of plaques than Ad-wt. Nonreplicating adenovirus Ad-ΔE1 did not develop any plaque except on E1 complementing cell line 293.

These ultramicroscopic features favor necrosis rather than apoptosis in that the cells do not exhibit the typical features of apoptosis, i.e., cell shrinkage, membrane blebbing, and membrane-bound apoptotic bodies. However, one of major characteristics of apoptosis, chromosome condensation, was observed in Ad-ΔE1B19 adenovirus–infected cells (Fig 5E). Therefore, it appears that the Ad-ΔE1B19 infection seems to have the capability of inducing both apoptosis as well as cellular necrosis in the same cells. Recent reports on murine polyomavirus infection showed specifically that the infected cells do undergo both apoptosis and necrosis, with cellular necrosis being the predominant event. Other viruses such as Newcastle disease virus,[24] human herpesvirus 7,[25] and infectious pancreatic necrosis virus[26] have also been shown to induce both apoptosis and necrosis in the same cell.

Ad-ΔE1B19 replicates as efficiently as wild-type adenovirus

Premature cell lysis by induction of apoptosis may lead to early viral release, possibly resulting in reduction of total production of viral progeny. Such a loss of viral production may reduce the efficacy of virotherapy for cancer treatment. Therefore, the effect of deletion of *E1B 19* kDa or presence of *E1B 55* kDa on viral production was examined. To examine and compare viral replication of each *E1B* mutant *versus* wild-type adenovirus, cells were infected with a specific adenovirus at an MOI of 1. Then the accumulation of viral particles in culture medium and cell fraction was determined by a plaque assay. As shown in Figure 6, total viral yields of Ad-ΔE1B19 and Ad-wt were similar in U343 cells, reaching a plateau after 5 days postinfection (Fig 6A). Ad-ΔE1B55 and Ad-ΔE1B19/55 virus yields did not reach a plateau until 8 days postinfection, and the total yield of virus was less than those of Ad-ΔE1B19 and Ad-wt. These results indicate that *E1B 55* kDa–containing adenoviruses, Ad-ΔE1B19 and Ad-wt, replicate very actively, whereas *E1B 55* kDa–deleted adenoviruses, Ad-ΔE1B55 and Ad-ΔE1B19/55, replicate less efficiently in the panel of cancer cells tested. However, the loss of *E1B 19* kDa function did not affect viral replication. Therefore, the efficient replication of adenovirus depends on the presence of *E1B 55* kDa, but not on the *E1B 19* kDa gene.

Ad-ΔE1B 19 adenovirus induced biggest plaques

Replication and viral spread from cell to cell was visualized in a plaque development assay. Monolayers of 293, Hep3B, and U343 cell lines were infected with five different adenoviruses, and plaque formation was monitored. Figure 7 shows a representative plaque assay with Ad-ΔE1, Ad-ΔE1B19, and Ad-wt. The size of plaques formed by Ad-ΔE1B19 was larger than that of Ad-wt, and more numbers of plaques were observed in Hep3B and U343 cells. However, all three adenoviruses (Ad-ΔE1, Ad-ΔE1B19, and Ad-wt) developed the same number and size of plaques in the 293 cells, which complement the *E1* gene in *trans*. The number of plaques formed in the Hep3B cells infected with *E1B* mutant adenoviruses was counted at 3, 5, and 8 days

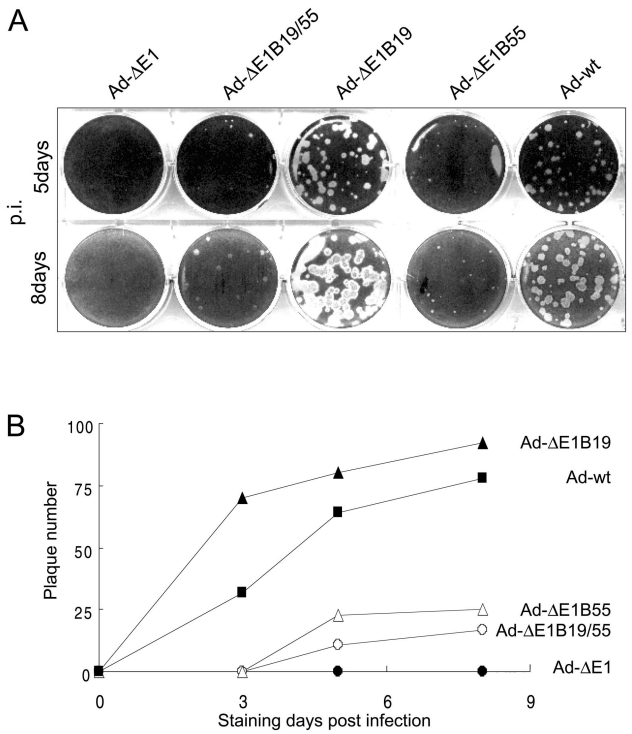

Figure 8 Plaque development assay for *E1B*-attenuated adenoviruses. Hep3B cells were infected with Ad-ΔE1, Ad-ΔE1B19/55, Ad-ΔE1B19, Ad-ΔE155, or Ad-wt. After 2 hours of adsorption period, plates were overlaid with agarose and incubated. After 3, 5, and 8 days postinfection, the agarose overlay was removed. The cells were then stained with crystal violet and counted. The plaques of Ad-ΔE1B19 and Ad-wt developed much faster than other *E1B* mutant adenoviruses, Ad-ΔE1B19/55 and Ad-ΔE155, but the final titers obtained for all virus stocks were similar.

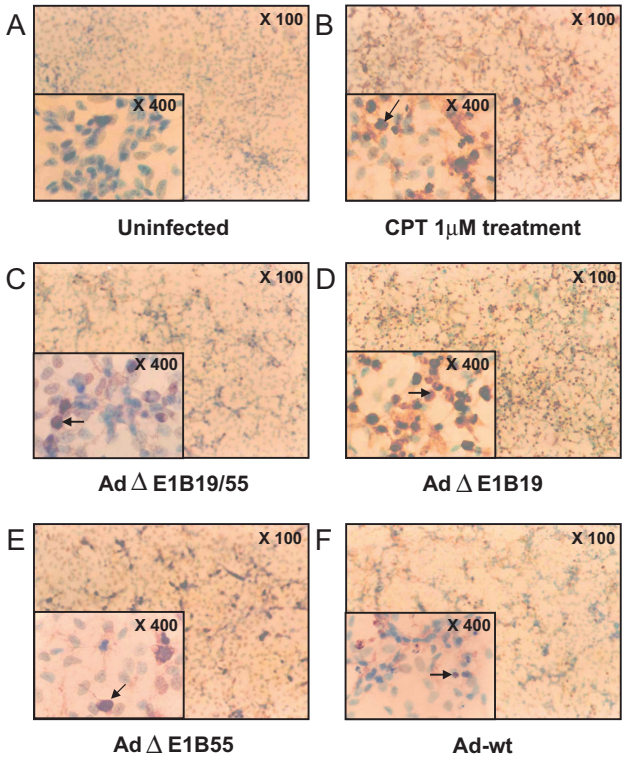

Figure 9 TUNEL staining in A549 cells. At 4 days after treatment without **(A)** or with 1 μM of CPT **(B)**, or infection with Ad-ΔE1B19/55 **(C)**, Ad-ΔE1B19 **(D)**, Ad-ΔE1B55 **(E)** or Ad-wt **(F)** at an MOI of 2, apoptotic cells were detected by labeling with DAB (3,3′-diaminobenzidine) using terminal deoxynucleotidyl transferase (counterstained with methyl green). Brown staining indicates positive staining for DNA strand breakage. Arrows indicate positive staining. Representative fields of two independent experiments are shown. Original magnification: ×100 and ×400.

after infection, and plotted in Figure 8. Consistent with results in Figure 7, Ad-ΔE1B19 showed enhanced plaque development when compared to other *E1B* mutants or wild-type adenovirus. With Ad-ΔE1B19, 92 plaques were observed at 8 days postinfection *vs* 78, 25, and 17 plaques for Ad-wt, Ad-ΔE1B55, and Ad-ΔE1B19/55, respectively (Fig 8B). Therefore, the rate of plaque development by infection with Ad-ΔE1B19 was faster than any other *E1B* mutant adenoviruses tested in this study, including the wild-type adenovirus. However, the other *E1B 19* kDa–attenuated adenovirus, Ad-ΔE1B19/55, developed plaques slowly and formed less number of plaques than Ad-wt, similar to Ad-ΔE1B55 (Fig 8B). Thus, Ad-ΔE1B19/55 and Ad-ΔE1B55 were not as effective as Ad-ΔE1B19 in promoting cell lysis, not only in CPE assay but also in the plaque development assay.

Cellular apoptosis is enhanced in the E1B 19 kDa–attenuated adenovirus-infected cells

To examine whether the attenuation of *E1B 19* kDa function could affect the cell death pathway, DNA integrity was monitored after viral infection. TUNEL and DNA fragmentation assays were used to evaluate the induction of apoptosis and tumor cell killing. A549 cells, infected with Ad-ΔE1, Ad-ΔE1B19, Ad-ΔE1B55, Ad-ΔE1B19/55, or Ad-wt at an MOI of 2, were evaluated for induction of apoptosis by TUNEL assay after 4 days postinfection. CPT (1 μM) was used as a positive control to induce apoptosis. Infection with Ad-ΔE1B19 induced the most potent apoptosis, showing that approximately 46.7% of the nuclei had a nicked DNA, whereas apoptosis induced by Ad-wt was nearly undetectable (4.3%) (Fig 9). The mean percentage of apoptotic cells induced by each adenovirus is summarized in Table 1. Ad-ΔE1B19/55 induced apoptosis in about 20% of the infected cells, whereas Ad-ΔE1B55 induced apoptosis at a very low rate (6.0%). Thus, DNA is degraded much faster in cells infected with Ad-ΔE1B19 or Ad-ΔE1B19/55 than in those infected with Ad-wt or Ad-ΔE1B55. These DNA breaks have been studied in more detail. The formation of DNA fragments consisting of multimers of approximately 180 bp has been regarded as a hallmark of apoptosis.[27] Figure 10 shows that *E1B 19* kDa–attenuated adenoviruses induced DNA ladder formation in U343 cells. DNA smear and the characteristic ladder were observed in cells treated with *E1B 19* kDa–attenuated adenoviruses (lanes 5 and 6) or CPT (lane 3), but not in the cells treated with Ad-ΔE1 (lane 4), Ad-ΔE1B55 (lane 7), or Ad-wt (lane 8). These results indicate that *E1B 19* kDa functions as an antiapoptotic molecule; thus, the elimination of *E1B 19* kDa protein induced apoptosis and accelerated cell death by adenoviral infection.

Suppression of growth of established tumors by the E1B mutant adenoviruses

We subsequently assessed the relative antitumor efficacy of E1B mutant adenoviruses in a human xenograft model (human hepatoma cell line, Hep3B, implanted in nude mice). When the subcutaneously implanted tumors reached 5 mm in diameter, tumors were injected intratumorally with Ad-ΔE1, Ad-ΔE1B19, Ad-ΔE1B55, Ad-ΔE1B19/55, or Ad-wt at a dose of 5×10^8 PFU per animal every 2 days for a total of three times. In tumors derived from the Hep3B cells, treatment with

Table 1 The mean percentage of apoptotic cells induced by each Ads

	Untreated	CPT (1 μM)	Ad–ΔE1B19/55	Ad–ΔE1B19	Ad–ΔE1B55	Ad-wt
Apoptotic cell ratio (%)	< 1.0	25.8	20.0	46.7	6.0	4.3

A549 cells were infected with Ad-ΔE1B19, Ad-ΔE1B55, Ad-ΔE1B19/55, or Ad-wt at an MOI of 10. Cells were harvested on day 4 after infection. The TUNEL assay was performed as described in Materials and methods. The number of brown stained cells per 100 cells was counted. Each of the indicated values reflects the mean of three independent experiments, and is expressed as the percentage of apoptotic cells.

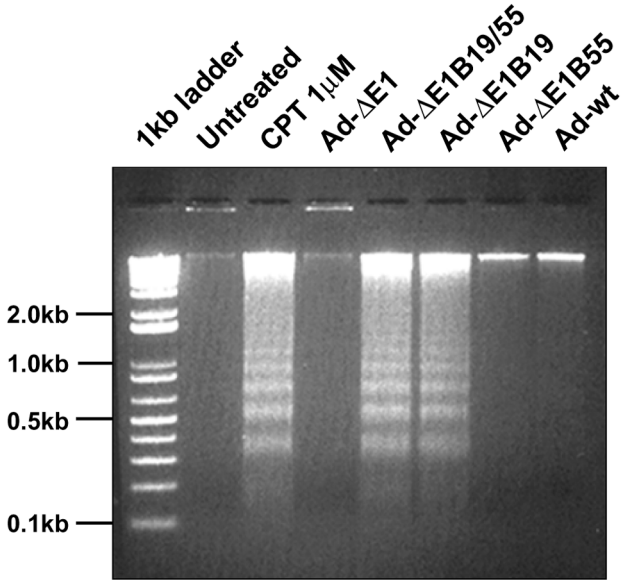

Figure 10 DNA fragmentation analysis. Confluent cultures of U343 cells were treated without (lane 2) or with CPT at a final concentration of 1 μM (lane 3). Cells were also treated with *E1B* mutant adenoviruses along with Ad-ΔE1 and Ad-wt as controls (lanes 4–8). The cells were harvested at 2 days posttreatment and the formation of DNA ladder was monitored by electrophoresis on a 2% agarose gel. DNA smear and the characteristic ladder can be seen in the cells treated with *E1B 19* kDa–attenuated adenoviruses (lanes 5 and 6) or CPT (lane 3), but not in those treated with Ad-ΔE1 (lane 4), Ad-ΔE1B55 (lane 7), or Ad-wt (lane 8).

the replicating adenoviruses significantly suppressed tumor growth when compared with control adenovirus, Ad-ΔE1. Control tumors grew very rapidly, with a greater than 33-fold increase in size by day 31 (day 1, 77.5±39 mm^3; day 31, 2541±592 mm^3), whereas *E1B*-attenuated adenoviruses

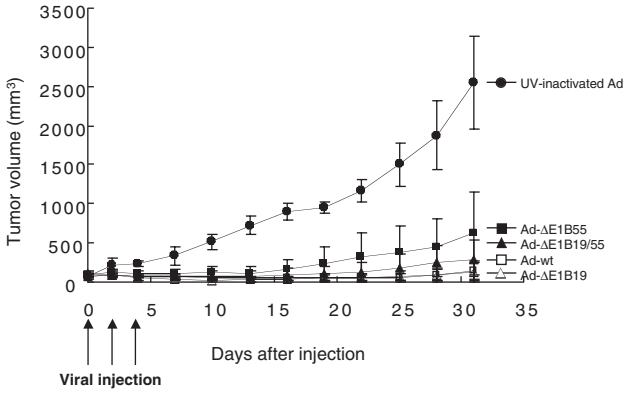

Figure 11 Efficacy of replicating adenoviruses against Hep3B tumors in female athymic nude mice. Tumors were established by subcutaneous implantation of 1×10^7 cells and allowed to grow to an average size of 60–70 mm^3. Animals with established tumors were randomized into five treatment groups of six animals each and treatment was initiated (day 1). Each group received intratumoral injection of adenovirus (5×10^8 PFU of adenovirus in 50 μL of PBS) on days 1, 3, and 5 (vertical arrows). Tumor growth was monitored on a 2- to 3-day interval by measuring the short length and long length of the tumor. Tumor volume was estimated on the basis of the following formula: volume = $0.523Lw^2$.

significantly suppressed the growth of Hep3B tumors (Ad-ΔE1B19, $\%\Delta T/\Delta C=2$; Ad-ΔE1B55, $\%\Delta T/\Delta C=21$; Ad-ΔE1B19/55, $\%\Delta T/\Delta C=8$, $P<.05$) (Fig 11). By day 10, Ad-ΔE1B19–treated tumors showed a $\%\Delta T/\Delta C$ of -11 (negative number signifies partial regression), with total tumor regression in one of the six mice tested. Ad-ΔE1B55– and Ad-ΔE1B19/55–treated tumors showed $\%\Delta T/\Delta C$ of 5 and -6, respectively, at this time period. The mouse showing regression has been followed for over 2 months without evidence of tumor regrowth. Similar results were obtained in the C33A tumor model with the subcutaneous injection of 1×10^7 C33A cells in nude mice (data not shown). Two animals from each treatment group were euthanized 7 days after the three sequential treatments, and the tumors were harvested for histological examination. Hematoxylin–eosin staining revealed that after infection with Ad-ΔE1B19, Ad-ΔE1B55, Ad-ΔE1B19/55, or Ad-wt, the remaining tumor mass consisted largely of necrotic tissue.

Minimal toxicity after intratumoral delivery of the replicating adenoviruses

To evaluate the replicating adenoviruses-related toxicity after intratumoral injection, histopathological changes in liver, kidney, and lung were examined for the two mice from each experimental group that were euthanized 7 days following three sequential intratumoral adenovirus administrations. No lesions were found in the liver, kidney, and lung in all animals examined (data not shown). To further document the toxicity by the replicating adenovirus treatment, blood samples were collected from animals 5 days after the last of the three sequential intratumoral treatments. Serum levels of liver enzymes, GOT, GPT, ALP, T-bili, and ALB were normal, and no significant differences were found among groups Table 2. Together, these results indicated that the intratumoral injection of replicating adenovirus is safe and well tolerated in mice and thus could be a promising approach for cancer therapy in humans.

Table 2 Blood analysis of Hep3B tumor-bearing mice treated with Ad-ΔE1B19 adenovirus

Description	Test	Unit	Untreated	Ad-ΔE1B19
Liver Function	GOT	U/L	115	84
	GPT	U/L	29	29
	ALP	U/L	360	237
	T-bili	mg/dl	0.4	0.4
	ALB	g/dl	2.8	2.6
Kidney Function	BUN	mg/dl	32.5	20.5
	CREA	mg/dl	1	0.6
Endocrine	CA	mg/dl	9.6	11.2
Electrolyte Test	Na	mEq/l	145	134
	K	mEq/l	7.6	5.5
	Cl	mEq/l	103	101
	GLU	mg/dl	161	253

Blood was collected from animals 5 days after the last of the three sequential intratumoral treatments. Serum levels of liver enzymes, GOT, GPT, ALP, T-bili, and ALB were normal, and no significant differences were found among groups.

Discussion

The importance of *E1B 55* kDa gene involved in p53 inactivation has been studied extensively. Similar activity has been reported for the *E4-ORF6* protein. Like *E1B 55* kDa, *E4-ORF6* binds p53 and antagonizes p53-mediated transactivation.[28,29] The *E4-ORF6* and *E1B 55* kDa proteins also regulate the function of p53 by affecting its half-life.[30–32] These functions of *E1B 55* kDa protein for p53 inactivation enabled the engineering of *E1B 55* kDa–deleted adenovirus for oncolytic adenovirus specific to *p53* mutant cancer cells. Additionally, the *E1B 55* kDa protein performs other important functions involved in viral replication. Late in adenovirus infection, large amounts of viral mRNA are accumulated in the cytosol, whereas cellular mRNA transport and translation are decreased.[19] Forming a physical and functional complex with *E4-ORF6*, the *E1B 55* kDa product mediates the accumulation of viral mRNA and inhibits the host cellular mRNA transport and translation.[20,33–35] Therefore, the loss of *E1B 55* kDa function endows tumor-selective replication and cell lysis, but this may compromise the efficiency of viral replication. To translate this work to a clinical application, this imperfection of the vector system could limit the therapeutic effect for cancer treatment.

The requirement of *E1B 55* kDa for efficient viral replication was also demonstrated in our study. In viral replication assay, only the Ad-ΔE1B19 adenovirus containing the *E1B 55* kDa replicated as efficiently as the wild type among the three *E1B*-attenuated adenoviruses. The other two mutant *E1B 55* kDa–deleted adenoviruses, Ad-ΔE1B55 and Ad-ΔE1B19/55, showed reduced viral replication in comparison to wild-type adenovirus. These lines of evidence demonstrate that *E1B 55* kDa gene is required in replicating adenoviral gene construct for efficient viral replication, which is critical for the therapeutic value of replicating adenovirus for cancer gene therapy.

In a CPE assay, the *E1B 19* kDa–inactivated adenovirus expressing *E1B 55* kDa had a greater ability to lyse cells than the *E1B 55* kDa–deleted counterpart Ad-ΔE1B19/55 and Ad-ΔE1B55 or the wild type. The other *E1B 19* kDa–attenuated mutant adenovirus, Ad-ΔE1B19/55, also showed more potent cell killing effect (about 5–50 times higher) than Ad-ΔE1B55. These findings support the conclusion that the deletion of antiapoptotic protein *E1B 19* kDa increased the rate of cell lysis. Sauthoff et al[23] previously reported that deletion of the *E1B 19* kDa gene may enhance the cytolytic effects of a replicating adenoviral vector, which is consistent with our results.

In respect to plaques morphology, the size of plaques formed by Ad-ΔE1B19 was larger than that of Ad-wt. Consistent with this, the total number of plaques appeared at an earlier postinfection period by Ad-ΔE1B19 infection and was higher than that of Ad-wt. However, Ad-ΔE1B19/55 did not form plaques as fast as Ad-ΔE1B19, even though both constructs have deletions of the *E1B 19* kDa protein. Formation of plaques is a net result of viral replication in the cells and the release of virus from infected cells. Thus, a possible explanation of how the *E1B 19* kDa–attenuated adenovirus, Ad-ΔE1B19, developed larger and more numbers of plaques than Ad-wt is either that they replicated more efficiently than wild-type Ad or that the progeny virions were released faster. From the result of viral replication assay (Fig 6), it was revealed that Ad-ΔE1B19 replicated at the same rate as that of wild-type adenovirus. Therefore, its more probable that the deletion of *E1B 19* kDa accelerated the efficient lysis of Ad-infected cells, resulting in Ad-ΔE1B19 forming the largest plaques that were faster to develop.

For the elucidation of the underlying mechanism responsible for the rapid cell killing of Ad-ΔE1B19 adenovirus, several assays detecting apoptosis were employed. The active induction of apoptosis by Ad-ΔE1B19 and Ad-ΔE1B19/55 adenoviruses was demonstrated by TUNEL and DNA fragmentation assay, and efficient cell lysis was shown by plaque-forming assay. These findings are in accordance with earlier studies in that infection with the *E1B 19* kDa mutant adenovirus caused the induction of cellular and viral DNA degradation and enhanced CPEs.[36–39] In support of these results, the *E1B 19* kDa protein has a well-established function of interfering with *E1A*-induced apoptosis, preventing premature death of the host cells.[40,41] In addition, the *E1B 19* kDa protein has been shown to inhibit the cytotoxic action of tumor necrosis factor α (TNF-α) or anti-Fas antibodies, both of which induce apoptosis.[42,43] Taken together, these results indicate that the deletion of the *E1B 19* kDa gene in replicating adenovirus induces *E1A*-mediated apoptosis as well as direct viral cytolysis, all of which ultimately lead to increased CPEs, rapid viral release, improved cell-to-cell spread, active induction of apoptosis, and efficient viral replication.

In recent clinical trials, tumor-selective replicating viruses were examined to assess its potential in enhancing the effect of known chemotherapeutic agents, cisplatin or 5-fluorouracil.[7,8] Results showed that the combination of tumor-selective replicating virus and either of these agents yielded a significantly better efficacy over treatment with chemotherapeutic agent alone. TNF-α[44] and adenovirus death protein (ADP)[45] have also been shown to enhance the efficacy of replicating adenoviruses. The *E1B 55* kDa gene–deleted replicating adenovirus, ONYX015, is currently in phase II clinical trial, being assessed for its ability to enhance the effect of cisplatin or 5-fluorouracil.[2,3] However, to date, all the replicating adenoviruses used in combination studies still retain the *E1B 19* kDa gene, which, as mentioned before, is a strong antiapoptotic agent. Because cell death induced by many chemotherapeutic agents is mainly driven by the apoptotic pathway, the *E1B 19* kDa gene may interfere with the mode of action of these agents, underestimating the value of these combination studies.

Our results confirmed previous reports that the deletion of *E1B 19* kDa function enhanced the cytolytic effect.[23] Moreover, Ad-ΔE1B19 adenovirus showed potent antitumor effect, suggesting that *E1A*-mediated apoptosis may be involved in addition to direct viral-induced cytolysis. The suppression of apoptosis by *E1B 19* kDa was also demonstrated by earlier reports in which the investigators reported that the *E1B 19* kDa protein efficiently prevents cisplatin-induced apoptosis.[46] Taken together, the replicating adenoviruses deleted in *E1B 19* kDa function may serve as an improved vector for anticancer gene therapy in

combination with other apoptosis-inducing modalities such as chemotherapeutic agents and radiation therapy.

Acknowledgments

We would like to thank all of our colleagues at Yosei Cancer Center, Seoul, Korea who have contributed to these studies. We thank J-H Son for his valueable contribution. This work was supported by grants from Ministry of Health & Welfare, Republic of Korea (HMP-01-PJI-PG3-20800-0115, C-O Yun) and Ministry of Commerce Industry and Energy, Republic of Korea (N03-990-5411-01-1-3, J-H Kim). Jaesung Kim is a graduate student sponsored by Brain Korea 21 Project for Medical Science, Yonsei University.

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